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Molecular cloning and expression of a unique receptor-like protein-tyrosine-phosphatase in the leucocyte-common-antigen-related phosphatase family

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Protein-tyrosine-phosphatases (PTPases) have been implicated in the regulation of certain tyrosine kinase growth factor receptors in that they dephosphorylate the activated (autophosphorylated) form of the receptors. In order to identify PTPases that potentially act on receptor targets in liver, we used the human leucocyte common antigen-related PTPase (LAR) cDNA [Streuli, Krueger, Hall, Schlossman and Saito (1988) J. Exp. Med. **168**, 1523–1530] and isolated two closely related transmembrane PTPase homologues from a rat hepatic cDNA library. Both PTPases had large extracellular domains that contained three immunoglobulin-like repeats and eight type-III fibronectin repeats. Both enzymes had tandem homologous PTPase domains following a single hydrophobic transmembrane domain. One sequence encoded the rat homologue of LAR. The second PTPase, designated LAR-PTP2, had 79 and 90 % identity with rat LAR in the respective cytoplasmic PTPase domains, with only 57 % sequence similarity in the extracellular domain. The catalytic domains of LAR and LAR-PTP2 prepared by bacterial expression were active in dephosphorylating a variety of phosphotyrosyl substrates but did not hydrolyse phosphoserine or phosphothreonine residues of labelled casein. Both enzymes exhibited rapid turnover numbers of 4–7 s^{-1} for myelin basic protein and 78–150 s^{-1} for derivatized lysozyme. LAR and LAR-PTP2 displayed similar PTPase activity towards the simultaneous dephosphorylation of receptors of intact insulin and epidermal growth factor from liver membranes. These data indicate that there is a family of LAR-related PTPases that may regulate the phosphorylation state of receptor tyrosine kinases in liver and other tissues.

INTRODUCTION

Protein phosphorylation is an essential mechanism in the control of cellular signalling through various physiological pathways. Increasing attention is being paid to reversible phosphorylation events involving protein tyrosine residues, which have been implicated in such diverse aspects of cellular control as signalling by insulin and other growth factors (Ullrich and Schlessinger, 1990; Myers and White, 1993), cell-cycle regulation (Gould et al., 1990) and the recognition of antigens by T-cells (Pingel and Thomas, 1989). Regulation of signalling through these pathways has been shown also to involve one or more cellular protein phosphatase enzymes with a marked or absolute specificity for protein tyrosine residues (PTPases; EC 3.1.3.48) (Fischer et al., 1991; Brautigan, 1992). In the case of the insulin holoreceptor, which retains its phosphorylation state and activation of its receptor kinase even after insulin is removed from the ligand-binding site, dephosphorylation of specific tyrosine residues is required to deactivate the intrinsic kinase activity of the receptor (Goldstein, 1993).

A number of full-length and partial PTPase sequences have now been cloned by several laboratories (Saito and Streuli, 1991; Fischer et al., 1991; Pot and Dixon, 1992). These enzymes comprise overlapping families of structurally related proteins with conserved cytoplasmic catalytic domains of approx. 260 amino acids that occur as single and tandemly related regions. The cloned enzymes may be divided into two broad categories: receptor-type, which have a general structure like a membrane receptor with a glycosylated extracellular domain, a single transmembrane segment and one or two conserved PTPase domains; and non-receptor-type, which lack a transmembrane segment and typically have a single PTPase domain and additional protein segments with various functional properties. Some of the non-receptor-type PTPases include PTPase1B (Guan et al., 1990; Chernoff et al., 1990; Brown-Shimer et al., 1990) and the T-cell PTPase (Cool et al., 1989), which have C-terminal hydrophobic domains that confer an association with the endoplasmic reticulum, PTPH1 and MEG (Yang and Tonks, 1991; Gu et al., 1991), which have N-terminal domains homologous to cytoskeleton-associated proteins, PTP1C (Shen et al., 1991; Plutzky et al., 1992; Yi et al., 1992) and SH-PTP2 (Freeman et al., 1992; Ahmad et al., 1993), which have two N-terminal SH2 domains that may promote their interaction with certain phosphotyrosine-containing substrates, PEP (Matthews et al., 1992), which has a proline-, glutamic acid-, serine- and threonine-rich ('PEST') domain at the C-terminus, and HePTPase (Zanke et al., 1992), which apparently lacks additional functional domains and appears to be a cytosolic PTPase expressed in lymphoid tissues.

The leucocyte common antigen (LCA) or CD45 (Trowbridge et al., 1991) was the first member of the family of receptor-type transmembrane PTPases to be identified. As CD45 was restricted in its expression to haematopoietic tissues, Streuli et al. (1988) used a CD45 cDNA probe to isolate a structurally related

Abbreviations used: DTT, dithiothreitol; PTPase, protein-tyrosine-phosphatase; LAR, leucocyte common antigen-related PTPase; LCA, leucocyte common antigen; MBP, myelin basic protein; N-CAM, neural cell adhesion molecule; RCML, reduced carboxamidomethylated maleylated lysozyme; poly(A)$^+$, polyadenylated.

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The sequences reported in this paper have been deposited in the EMBL/GenBank DDBJ Nucleotide Sequence Databases under the following accession numbers: rat LAR, L11586; rat LAR-PTP2, L11587; rat LAR-PTP2B, L12329.

PTPase homologue from a human placental library, called the leucocyte common antigen-related phosphatase (LAR), which is expressed in liver and a variety of cell lines and tissues (Streuli et al., 1988; Goldstein et al., 1991). Additional receptor-type PTPases have been cloned by several laboratories from mammalian tissue sources including LRP, for LCA-related phosphatase (also termed RPTP-α) (Matthews et al., 1990; Sap et al., 1990; Krueger et al., 1990), RPTP-β, RPTP-δ, RPTP-ϵ (Krueger et al., 1990), RPTP-μ (Gebbink et al., 1991) and RPTP-ζ (Krueger and Saito, 1992). Krueger et al. (1990) have also provided evidence that LAR and RPTP-δ have a close phylogenetic relationship and may comprise a subfamily of transmembrane PTPases.

Our laboratory is interested in isolating PTPases that are expressed in liver and other insulin-sensitive tissues in order to characterize their potential physiological role in the regulation of receptor tyrosine kinase activity and growth factor action. As the bulk of insulin-receptor dephosphorylating activity is localized to a particulate fraction of liver and hepatoma cells, we used a cDNA fragment from the human transmembrane PTPase, LAR, as a probe for molecular cloning of additional related homologues at reduced stringency in a rat liver cDNA library. A unique transmembrane PTPase homologue in the LAR family was isolated and characterized. The activity of the cytoplasmic domains of the cloned PTPases was also evaluated using a bacterial expression system and several tyrosine-phosphorylated protein substrates including intact receptors for insulin and epidermal growth factor.

EXPERIMENTAL

Materials

A mixed oligo(dT) and random hexamer-primed cDNA library in λZAPII prepared from the liver of a male Sprague–Dawley rat was obtained from Stratagene (La Jolla, CA, U.S.A.). DNA restriction endonucleases and modifying enzymes were purchased from New England Biolabs (Beverly, MA, U.S.A.) or Promega (Madison, WI, U.S.A.). Oligonucleotides were purchased from Oligos Etc. (Wilsonville, OR, U.S.A.). *Thermus aquaticus* DNA polymerase was purchased from Perkin–Elmer Cetus (Norwalk, CT, U.S.A.). Modified T7 DNA polymerase for sequencing was obtained from U.S. Biochemical (Cleveland, OH, U.S.A.). [α-[^{35}S]thio]dATP and [γ-^{32}P]ATP were from New England Nuclear (Beverly, MA, U.S.A.). The pGEX-KG expression vector was purchased from the American Type Culture Collection. Myelin basic protein (MBP) and reduced carboxamidomethylated maleylated lysozyme (RCML) were from Sigma (St. Louis, MO, U.S.A.).

cDNA cloning

One million phage plaques of the rat liver cDNA library were hybridized (Sambrook et al., 1989) at reduced stringency in a solution containing 40% (v/v) formamide, 0.1% (w/v) Ficoll, 0.1% (w/v) poly(vinylpyrrolidone), 0.1% (w/v) BSA, 5 mM EDTA, 0.1% (w/v) SDS, 100 μg/ml denatured salmon sperm DNA, 750 mM NaCl and 50 mM NaH_2PO_4, pH 7.4, at 35 °C with a labelled cDNA (Feinberg and Vogelstein, 1983) encoding all but the extremes 5′ end of the human LAR sequence (kindly provided by Dr. Haruo Saito, Dana Farber Cancer Institute, Boston, MA, U.S.A.). Filters were washed at 50 °C in buffer containing 75 mM NaCl and 0.1% (w/v) SDS in 7.5 mM trisodium citrate, pH 7.0. Twenty-four positive clones were obtained and plaque-purified by secondary screening with the same LAR cDNA probes. Insert-bearing pBluescript phagemids were excised from the λZAPII clones using the helper phage R408 and plasmids were prepared by infection of XL-1 Blue *Escherichia coli* host cells (Stratagene Cloning Systems, 1988). In a tertiary screening involving hybridization of plasmid DNA, slot-blots were used to identify 15 clones that hybridized strongly with the LAR domain probe (*Hin*dIII/*Eco*RI segment). Double-stranded sequencing was then performed on both strands of the cDNA clones using the chain-termination method of Sanger et al. (1977) with T3 and T7 promoter primers, or synthetic 17-mer oligonucleotides derived from previously obtained sequences, and modified T7 DNA polymerase (U.S. Biochemical). For large (> 2 kb) inserts, nested deletions were prepared for sequencing each strand as described previously (Goldstein and Dudley, 1990).

Additional cDNA segments were obtained by cDNA walking at high stringency in a rat brain cDNA library in λGT10 (Stratagene). The remaining segments of the PTPase cDNAs were cloned by an anchored reverse transcription PCR technique (Frohman et al., 1988) employing poly(dA) tailing with terminal transferase and amplification with Vent thermostable polymerase (New England Biolabs).

Analysis of sequence homology

Sequences were tested for homology with the translated version of the GenBank database, and amino acid alignments were performed using programs in the EuGene package, both accessed through the Molecular Biology Computer Research Resource at Boston University.

Blot hybridization analyses

Male Sprague–Dawley rats weighing 150–175 g were obtained from Taconic Farms (Germantown, NY, U.S.A.) and had free access to food and water before being killed. RNA was prepared from various rat tissues by the single-step method (Chomczynski and Sacchi, 1987), and Northern-blot analysis was performed with poly(A)$^+$ RNA as described previously (Goldstein et al., 1987). A blot containing 2 μg of poly(A)$^+$ RNA from multiple rat tissues was also hybridized according to directions supplied by the manufacturer (Clontech). Genomic DNA was isolated from rat liver (Ludwig et al., 1988) and after digestion with the indicated restriction enzyme, 5 μg was subjected to electrophoresis in a 0.8% agarose gel in buffer consisting of 50 mM boric acid, 1 mM EDTA and 50 mM Tris/HCl, pH 7.0, denatured, and transferred to nitrocellulose. Filters were hybridized with cDNA fragments labelled by random hexamer priming (Feinberg and Vogelstein, 1983).

Purification of bacterially expressed PTPase catalytic domains

For enzyme studies, the entire cytoplasmic domains of LAR and LAR-PTP2 were expressed as glutathione S-transferase fusion proteins in the pGEX-KG vector system using standard methods similar to those described by Hashimoto et al. (1992b). The final subcloned products for LAR and LAR-PTP2 expression were checked by restriction mapping and sequencing of plasmid inserts.

Recombinant fusion proteins were expressed by transformation of *E. coli* HB101 with the pGEX vectors, and induction of protein synthesis and lysis of bacteria were performed as described (Hashimoto et al., 1992b). Fusion proteins were isolated by glutathione–agarose affinity chromatography, cleaved with thrombin, and purified free of glutathione S-transferase over the glutathione–agarose column (Guan and Dixon, 1991).

Dephosphorylation of receptors for insulin and epidermal growth factor

Growth factor receptors were enriched from a solubilized membrane fraction of rat liver by wheat germ agglutinin–agarose chromatography (Kasuga et al., 1985). Receptors for insulin and epidermal growth factor (4 μg of protein of the lectin column eluate) were labelled in a 0.4 ml reaction mixture containing 1 μM insulin, 5 μg/ml epidermal growth factor, 5 mM $MnCl_2$, 25 μM ATP, 200 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) and 0.05% (v/v) Triton X-100 in 50 mM Hepes, pH 7.6, at 4 °C for 60 min. Unincorporated [^{32}P]ATP was removed on a Bio-Gel P6 spin column, and 25 μl portions of the labelled receptors were incubated with 1.5 μg of recombinant PTPase cytoplasmic domain protein in a 150 μl reaction mixture containing 1 mM dithiothreitol (DTT) and 10 mM EDTA in 50 mM Hepes, pH 7.4, at 30 °C. After the indicated period of time, a 5× concentrated gel sample buffer was added and 30 μl of the reaction volume was subjected to electrophoresis in gels containing SDS and 7.5% polyacrylamide (Laemmli, 1970) and analysed by autoradiography.

Dephosphorylation of additional phosphoprotein substrates

MBP and RCML were phosphorylated on tyrosine residues with partially purified recombinant insulin receptors noted above and purified as described by Tonks et al. (1991a,b). Dephosphorylated bovine casein (Sigma) was phosphorylated with the catalytic subunit of cyclic AMP-dependent protein kinase (Promega), under conditions suggested by the supplier. The ^{32}P-labelled casein was purified by precipitation with trichloroacetic acid and dialysed into PTPase reaction buffer. After incubation at 30 °C for up to 10 min, ^{32}P released was measured by the addition of 0.90 ml of acidic charcoal mixture [0.9 M HCl, 90 mM sodium pyrophosphate, 2 mM NaH_2PO_4, 4% (v/v) Norit A], sedimentation of the precipitate in a Microfuge and determination of radioactivity in 0.5 ml of the supernatant (Krueger et al., 1990). For kinetic studies using the purified recombinant PTPases, reactions were terminated at an appropriate time for the determination of initial reaction rates.

RESULTS

cDNA cloning

Using the human LAR cDNA (Streuli et al., 1988) to probe a rat liver cDNA library at reduced stringency, eight clones were identified after a tertiary screening with a probe derived from a limited region of the intracellular LAR domain (*Hin*dIII/*Eco*RI fragment). Among these initial cDNA clones, analysis of overlapping cDNA segments revealed that two sequences were present. One of the cDNAs was represented by seven cDNA clones. This sequence was nearly identical with the human LAR cDNA sequence and represents its rat homologue (see below). Additional segments of the full-length rat LAR cDNA were then compiled from additional screenings of rat liver and brain cDNA libraries. The remaining 546 bp of the 5′ region was obtained by an anchored PCR technique with rat liver mRNA (Frohman et al., 1988).

One unique cDNA insert from the initial rat liver library screening (clone 12 in Figure 1) had high regional homology to the intracellular portion of the LAR cDNA but appeared to encode a different gene product. We have termed this cDNA LAR-PTP2. An additional segment of this relatively rare hepatic cDNA (Figure 1, clone 9-1) was isolated by cDNA walking with an additional one million plaques of the same rat liver library using a cDNA segment derived from the 5′ end of clone 12. Additional segments of the full-length rat LAR-PTP2 cDNA were then compiled from additional screenings of a rat brain cDNA library (clone RB22-2), and the remaining 882 bp of sequence at the 5′ end was obtained by using the anchored PCR technique with rat brain mRNA (Frohman et al., 1988). Interestingly, one cDNA clone for LAR-PTP2 from the rat brain library screening (RB22-2) had a deleted internal segment of 1086 bp that is flanked by a sequence that exactly matches regions of clone 12-1, suggesting that the *LAR-PTP2* gene may generate alternatively spliced mRNA products in a tissue-specific fashion (see below).

The 6545 bp cDNA sequence of rat LAR contained a single long open reading frame of 5697 bp. The putative translational start site contains a well-conserved consensus sequence as defined by Kozak (1991). The downstream methionine residue (Met-11) was postulated to be the N-terminal residue of the proprotein for human LAR (Streuli et al., 1988), but in the rat sequence this has a less conserved consensus sequence for translation initiation. Furthermore, nine of the first ten residues of the postulated rat LAR precursor are identical with the human sequence, suggesting

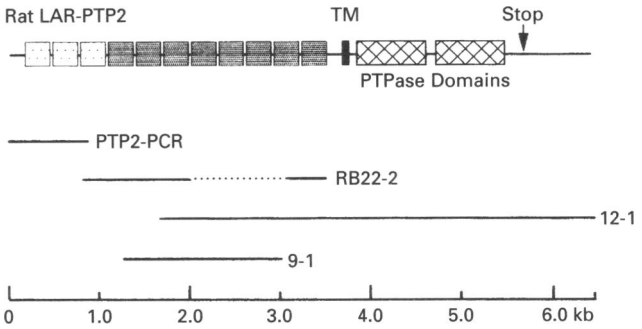

Figure 1 Diagram of cDNA clones encoding LAR-PTP2

The numbered inserts were isolated from a rat liver library and the insert designated RB was from a rat brain library. The 5′ sequences were obtained by anchored cDNA amplification and labelled PCR in the diagram. The extracellular domains include three regions with homology to immunoglobulin repeats (light stippling) and eight regions with fibronectin type III homology (dark stippling), as described in the text. The transmembrane domain (TM) and the two tandem homologous PTPase domains in the cytoplasmic region are also shown. The dotted line in clone RB22-2 encoding rat LAR-PTP2 indicates absent sequences that are probably due to alternative splicing of this rat brain transcript.

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rPTP2:      GSLLARWEPPADAAEDPVLGYRLQFGR.E      PGAETALTLQGLRPETAYELRVRAHTRRGPGP
             SLL+ W PP+      + GY L +   E      P+  T++ L GLRP ++Y ++V+A   RG GP
mN-CAM: 931 TSLLLHWQPPLSHNGV.LTGYLLSYHPVE 873 PANTTSAILSGLRPYSSYHVEVQAFNGRGLGP
            ++LL+W+PP    G  L GY L Y+  +       +    + L GL+P ++Y ++V+A   +G+GP
rLAR:       HTALLQWHPPKELPGE.LLGYRLQYRRAD      VTGDVHLTLLGLKPDTTYDIKVRAHTSKGAGP

rPTP2:      VVVRTDEDVPSAPPRKVEAEALNATAIRVLWRSPTPGRQHGQIRGYQV
            + V T  DVPSAPP+ V  E +N+ +I+V W  P  G Q G I GY +
hDCC: 613   ITVVTLSDVPSAPPQNVSLEVVNSRSIKVSWLPPPSGTQNGFITGYKI
            + V T  DVPS+PP+ V +E +NS ++ VSW  P    Q G I GY +
rLAR:       VLVRTDEDVPSGPPRKVEVEPLNSTAVHVSWKLPVPNKQHGQIRGYQV
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Figure 2 Alignment of segments of the extracellular domain of rat LAR-PTP2 (rPTP2) and rat LAR (rLAR) with homologues in the N-CAM family, including mouse N-CAM (mN-CAM) and a putative human colorectal tumour suppressor protein DCC (hDCC) (Fearon et al. 1990)

Between the amino acid sequence, identical residues are indicated with a capital letter and conservative substitutions are shown by a (+). In a few cases, a single amino acid gap has been introduced for alignment (.).

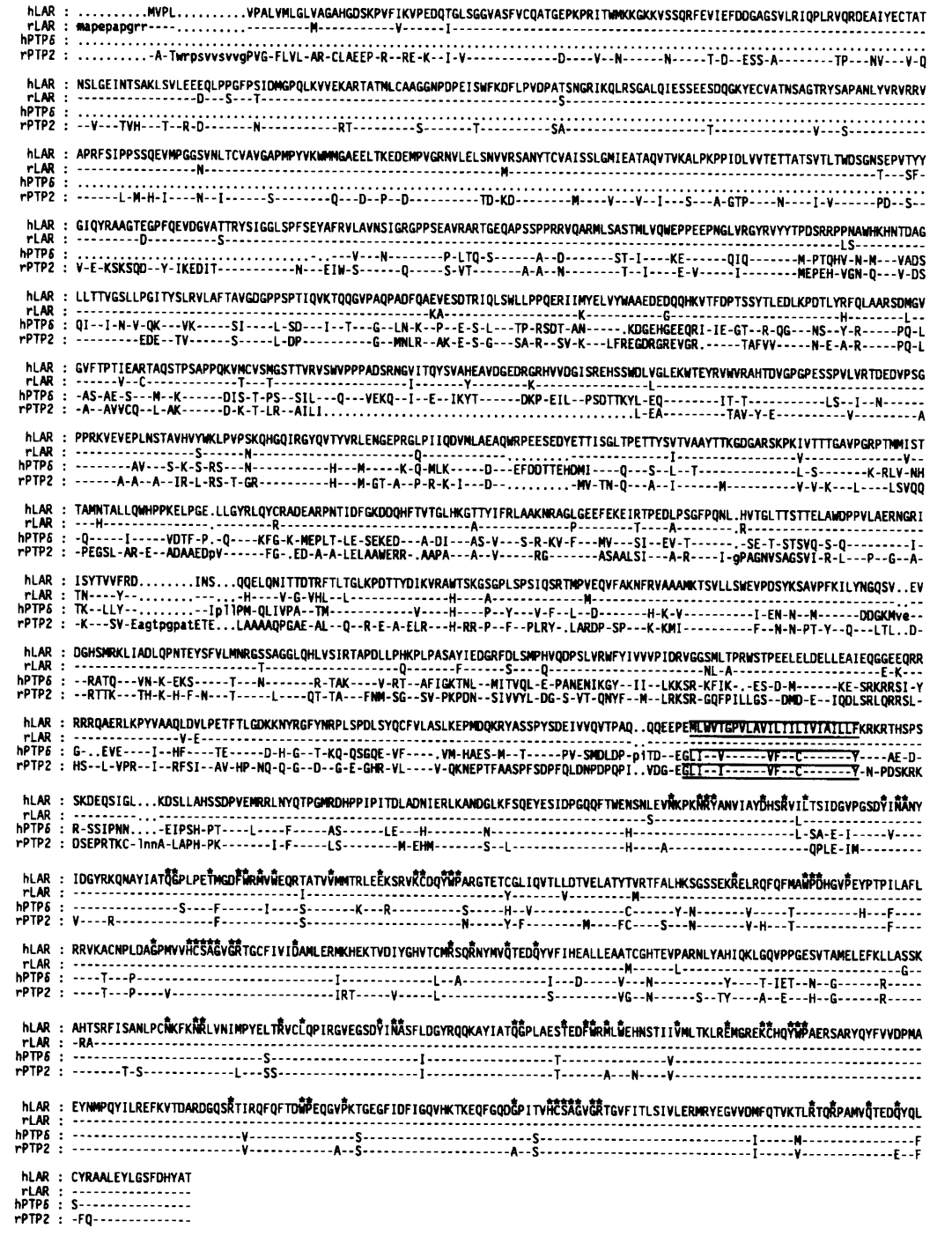

Figure 3 Alignment of amino acid sequences deduced from the cDNAs for human LAR (hLAR), rat LAR (rLAR), human PTP-δ (hPTPδ) and rat LAR-PTP2 (rPTP2) as determined by the algorithms in the EuGene DNA analysis programs

As shown, human PTP-δ contains the available partial sequence data (Krueger et al., 1990). The sequence comparison is presented as follows: upper case, aligned non-identical residues; lower case, unaligned residues; ———, aligned identical residues; ...., a gap introduced for optimal alignment. The transmembrane domains are highlighted by underlining. The regions containing the tandem intracellular PTPase domains are indicated, along with residues that are conserved in the PTPase catalytic domains which are highlighted by an asterisk over the human LAR sequence (Krueger et al., 1990).

that the actual translation start site for the human LAR cDNA may be the more proximal methionine residue. Using the algorithm of von Heijne (1986), the signal peptide cleavage site is predicted to occur between Ala-27 and His-28. Thus the mature protein contains 1871 amino acids, including an extracellular domain of 1224 residues, 24 hydrophobic residues comprising a single transmembrane segment (Met-1252–Phe-1275) and a cytoplasmic domain of 623 amino acids. This sequence is identical with the partial rat LAR cDNA sequence (854 amino acids) reported by Pot et al. (1991) with the exception

of five positions where Thr-1073, Ile-1434, Gly-1639, Arg-1643 and Ala-1644 in our sequence are replaced by Ser, Thr, Asn, His and Thr respectively in the sequence of Pot et al. (1991).

The cDNA sequence of rat LAR-PTP2 consisted of 6469 bp, with a single long open reading frame of 5592 bp. There is a single in-frame termination codon upstream (nt116) of the putative translational start site (nt185) which contains a well-conserved initiation sequence; the signal-peptide-cleavage site is predicted to occur between Gly-26 and Cys-27. Thus the mature protein contains 1837 residues, which includes an extracellular domain of 1186 residues, 25 hydrophobic residues in the single transmembrane domain (Gly-1213–Tyr-1237) and a cytoplasmic domain of 626 amino acids. The alternative sequence of the LAR-PTP2 cDNA found in clone RB22-2 from rat brain shortens the extracellular domain by 362 amino acids (Figure 1). We have termed the protein resulting from the translation of this alternatively spliced mRNA LAR-PTP2B.

Homology comparisons with the deduced amino acid sequences of rat LAR and LAR-PTP2 were performed against the translated version of the GenBank database (Figure 2). Similarly to data reported for other receptor-type PTPases (Streuli et al., 1988), the extracellular domain of both LAR and LAR-PTP2 had striking regional homologies to certain fibronectin domains (Kornblihtt et al., 1985) and portions of the neural cell adhesion molecule (N-CAM) structure (Cunningham et al., 1987). In addition, both rat LAR and LAR-PTP2 have marked segmental homology to a putative colorectal tumour suppressor protein that has structural similarities to the N-CAM family (Fearon et al., 1990), but whose function is unknown.

LAR and LAR-PTP2 proteins have a high degree of amino acid identity in each of the defined functional domains (Figure 3). The rat LAR homologue had 95.3% overall sequence identity to human LAR with even higher identities in the transmembrane (100%) and cytoplasmic (98.2%) PTPase domains. PTP-2 had an overall structural organization that was similar to that of human LAR. These features included three immunoglobulin-type cysteine repeats near the N-terminus with high conservation of the consensus D-X-A(G)-X-Y-X-C sequence around the second cysteine residue of each repeat in both LAR and LAR-PTP2 (Cunningham et al., 1987). Both proteins also exhibited eight fibronectin type-III repeats in the extracellular domain. The alternatively spliced segment of the rat brain cDNA encoding LAR-PTP2B effectively removes the fourth–sixth fibronectin type III repeats. Despite their overall conservation of sequence identity, the rat LAR and LAR-PTP2 sequences diverge significantly in many areas of the extracellular domain, especially near the transmembrane segment and in the proximal portion of the cytoplasmic domain near the membrane. Interestingly, in the internal portion, the amino acid identity between rat LAR-PTP2 and rat LAR is greater in the second PTPase region (89.6%) than in the proximal domain (78.7%), as has been noted for other tandem-domain PTPases (Krueger et al., 1990). In comparison with the published partial sequence for human PTP-δ, a human transmembrane PTPase phylogenetically related to LAR (Krueger et al., 1990), LAR-PTP2 has 59.3% overall identity. Interestingly, the extracellular domain of rat LAR-PTP2 was more closely related to LAR than to human PTP-δ (56.7% identity compared with 40.2%). These data indicate that there is a family of LAR-related receptor-like PTPases that may have arisen by gene duplication. Differences in the overall sequence similarity between LAR-PTP2 and human PTP-δ, however, suggest that it is not simply the rat homologue of human PTP-δ. The substantial sequence variation in the extacellular domain also implies that it may have a functional role in the rat that is different from PTP-δ in human tissues.

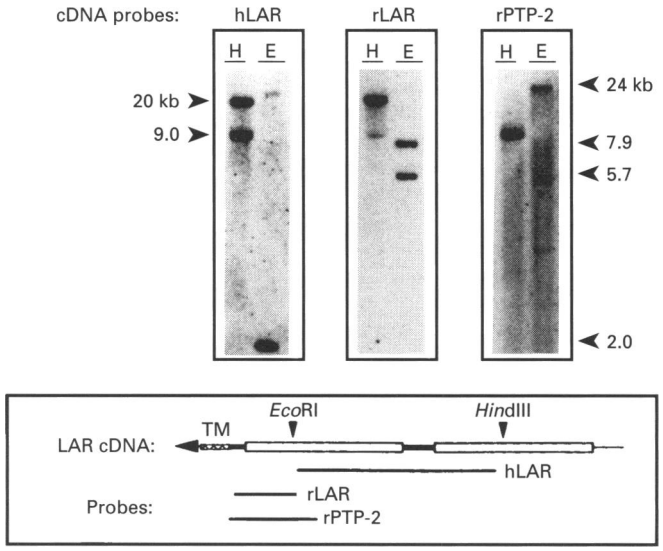

Figure 4 Southern-blot analysis of rat genomic DNA digested with either *Hin*dIII (H) or *Eco*RI (E) and hybridized with cDNA probes from human LAR (hLAR), rat LAR (rLAR) or rLAR-PTP2 as indicated

The sequence origin of the cDNA probes is shown schematically in the lower panel; the hLAR probe was an *Eco*RI/*Hin*dIII fragment, the rLAR probe was an *Eco*RI fragment of clone 18-2, and the rLAR-PTP2 probe was an *Apa*I fragment of clone 12. Note that the *Eco*RI site is converted between the human and rat LAR cDNAs but is not present at that position in the rLAR-PTP2 cDNA sequence.

Blot hybridization studies

Southern-blot analysis with rat genomic DNA indicated that the human LAR domain probe (*Hin*dIII/*Eco*RI fragment) hybridizes only with two discrete fragments of *Hin*dIII-digested rat DNA under the reduced stringency conditions used in the library screening (Figure 4). Interestingly, the 20 kb genomic fragment hybridizes only with a cDNA segment from the PTPase domain of the rat LAR clone, whereas the 9.0 kb fragment hybridizes predominantly with the cDNA probe derived from an identical region of the conserved first PTPase domain of LAR-PTP2. These data suggest that the probe used in the initial library screening cross-reacts with genomic segments derived from the coding regions of the two closely related PTPase genes that have been isolated. With *Eco*RI-digested genomic DNA, the human LAR cDNA probe hybridizes to different genomic fragments from the rat LAR cDNA probe because the converted *Eco*RI site in the cDNA prevents cross-hybridization between the restricted genomic exons and the probe cDNA sequences. The human LAR and LAR-PTP2 probes each hybridize with a large 24 kb fragment of *Eco*RI-digested rat genomic DNA, however, since there is potential sequence overlap between these homologous probe sequences. Furthermore, hybridization of *Eco*RI-digested rat genomic DNA with cDNA segments of rat LAR and LAR-PTP2 taken from identical regions of the first PTPase domain revealed distinct simple patterns, providing further evidence that each of these cDNA sequences corresponds to unique single-copy genes.

The expression of rat LAR and LAR-PTP2 was evaluated by Northern-blot analysis using poly(A)$^+$ RNA from several rat tissues. In preliminary studies using the probes from the cytoplasmic domain indicated in Figure 4, rat LAR cDNA hybridized to a major mRNA transcript of 8.0 and a minor band of 5.3 kb in liver. The 8.0 kb mRNA was also expressed in kidney and to

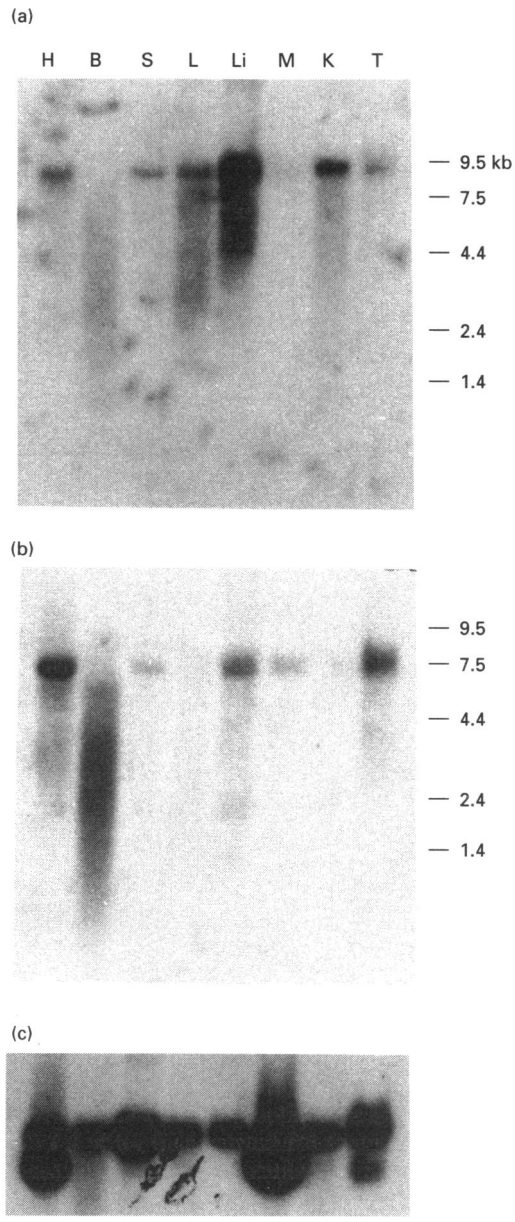

Figure 5 Northern-blot analysis of mRNA from several rat tissues using rat LAR (a), LAR-PTP2 (b) and β-actin (c) cDNAs as probes

Electrophoresis in 1% agarose gels containing 3% (v/v) formaldehyde was performed with 2 μg of poly(A)$^+$ RNA from rat heart (H), brain (B), spleen (S), lung (L), liver (Li), skeletal muscle (M), kidney (K) and testis (T). The RNAs were transferred to nylon filters and hybridized at high stringency according to the manufacturer's instructions (Clontech). For these blots, the cDNA probes were the entire cytoplasmic domains of LAR and LAR-PTP2 and a 2 kb human β-actin control probe used sequentially on the same blot. The positions of RNA size markers are also indicated.

a lesser extent in brain and spleen. These results are essentially in agreement with Northern-blot data from human cell lines (Streuli et al., 1988). Using the entire rat LAR cytoplasmic domain as a probe, hybridization of mRNA from multiple rat tissues showed predominant expression of LAR in liver as an 8.0 kb mRNA, with two minor bands at ~ 5.0 and ~ 4.6 kb (Figure 5). In decreasing order, expression of the 8.0 kb transcript was also seen in kidney, heart, lung, spleen, testis and skeletal muscle, with no apparent expression of the smaller mRNA species. Interestingly, brain RNA contained a transcript that co-migrated with the 4.6 kb liver mRNA for LAR and a diffuse signal suggesting additional size mRNAs. The diffuse signal might also suggest cross-reactivity with other related PTPases in brain, but this is less likely given the high stringency of the hybridization and the lack of cross-reactivity on these blots between LAR and its close homologue LAR-PTP2 (see below). The hybridization signal of the same blot with a probe for β-actin attests to the integrity of the RNA preparation.

In contrast, Northern-blot analysis of the same blot using a cDNA probe encompassing the entire cytoplasmic domain of LAR-PTP2 revealed a single mRNA transcript of 7.3 kb with predominant expression in heart, testis and liver, and lesser expression in skeletal muscle, spleen, brain and kidney (Figure 5). By examining the superimposed blots for LAR and LAR-PTP2, the high-molecular-size transcript for LAR is measurably larger than the LAR-PTP2 mRNA. In the blot shown in Figure 5 and in independent Northern-blot hybridizations (not shown), a major transcript for LAR-PTP2 was also observed at 6.0 kb which was expressed to a high level only in brain tissue. Additional mRNA species for LAR-PTP2 are apparent in the range of ~ 2.6 to 4.4 kb. Reverse transcription and cDNA amplification of brain RNA with primers flanking the alternatively spliced domain of LAR-PTP2 revealed products of ~ 1.9 kb as well as smaller products in the 200–400 bp range, providing further evidence that both forms of the LAR-PTP2 mRNA are expressed in brain tissue (results not shown). The presence of multiple splice forms of the LAR-PTP2 transcripts may also account for the broad multiple bands we and others have observed on Northern-blot analysis of PTP-2 in brain (Yan et al., 1993; Pan et al., 1993).

Catalytic activity of LAR and LAR-PTP2 cytoplasmic domains

In order to examine the enzyme activity of the catalytic domains of the cloned PTPases, the entire cytoplasmic domains of LAR and LAR-PTP2 were expressed as recombinant glutathione S-transferase fusion proteins using the pGEX-KG vector. The PTPase domains were cleaved from the fusion product and purified essentially to homogeneity. Both protein domains migrated at the expected molecular size of approx. 72 kDa (results not shown).

The catalytic activity of the recombinant PTPase domains was tested *in vitro* towards the simultaneous dephosphorylation of intact autophosphorylated receptors for insulin and epidermal growth factor prepared from rat liver membranes (Figure 6). In these studies, the dephosphorylation of the 95 kDa insulin receptor β-subunit and the 170 kDa epidermal growth factor receptor protein was assessed by gel electrophoresis and autoradiography of the receptor polypeptides after incubation with the purified PTPase enzymes. In the control receptor preparation, minimal dephosphorylation of the intact receptors was observed during the incubation period. After incubation with recombinant LAR or LAR-PTP2, both autophosphorylated receptors were simultaneously dephosphorylated with no significant difference in the relative PTPase activity towards the dephosphorylation of either insulin or epidermal growth factor receptors.

The purified LAR and LAR-PTP2 cytoplasmic domains were also used to study catalytic properties of their encoded PTPases. Casein, phosphorylated with the catalytic subunit of cyclic AMP-dependent protein kinase, was not dephosphorylated by either LAR or LAR-PTP2 over background (not shown). As a control, calf intestinal alkaline phosphatase (Promega) rapidly de-

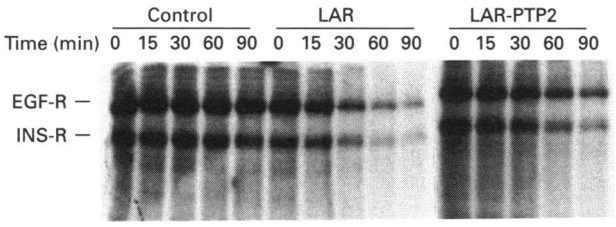

Figure 6 Simultaneous dephosphorylation of intact autophosphorylated receptors for insulin and epidermal growth factor by the catalytic domains of rat LAR and LAR-PTP2

Purified recombinant cytoplasmic domains of LAR and LAR-PTP2 were used to dephosphorylate insulin and epidermal growth factor receptors as described in the Experimental section. After the indicated period of time, a stop solution was added and the receptors were analysed by gel electrophoresis and autoradiography. The positions of the 95 kDa insulin receptor β-subunit (INS-R) and the 170 kDa epidermal growth factor receptor (EGF-R) are shown.

Table 1 Effect of various compounds on the catalytic activity of purified LAR and LAR-PTP2

The data shown represent the percentage of control dephosphorylation of MBP or RCML in the absence of effector; the mean value of at least two experiments is given. nd, Not determined.

		LAR activity (%)		LAR-PTP2 activity (%)	
Addition	Concentration	MBP	RCML	MBP	RCML
None		100	100	100	100
Na_3VO_4	100 μM	2.7	51	0	10
	1 mM	2.5	21	0	0
Ammonium	10 μM	48	70	1.5	12
molybdate	100 μM	19	0	nd	nd
Spermine	2 mM	159	75	124	78
EDTA	5 mM	123	62	131	58

Table 2 Kinetic parameters of purified LAR and LAR-PTP2 cytoplasmic domains

The specific activity is expressed as nmol of P_i released/min per mg of protein and is the initial linear reaction rate using 0.2 μg of each PTPase for the reaction with MBP and 0.02 μg each for RCML. $V_{max.}$ is expressed as nmol of P_i released/min and was determined by Michaelis–Menten kinetics. The turnover number is nmol of P_i released/min per nmol of enzyme.

	LAR		LAR-PTP2	
Parameter	MBP	RCML	MBP	RCML
Specific activity	706	5550	350	3720
K_m (μM)	4.35	7.93	2.94	3.34
$V_{max.}$	1.18	2.50	0.643	1.33
Turnover number	424	8990	233	4780

phosphorylated more than 50% of the labelled casein substrate. In contrast, tyrosine-phosphorylated MBP and RCML were actively dephosphorylated by both PTPases (see below), indicating their catalytic specificity for protein phosphotyrosine residues.

A series of effectors that have previously been shown to stimulate or inhibit various PTPases was tested with the LAR and LAR-PTP2 enzymes using both MBP and RCML as substrates (Table 1). Sodium vanadate was inhibitory to both enzymes, although the K_i (IC_{50}) was substantially higher for LAR using RCML as substrate (100 μM) than for MBP with either enzyme or for RCML with LAR-PTP2. Another PTPase inhibitor, ammonium molybdate, was inhibitory to both enzymes but LAR appeared less sensitive than LAR-PTP2 with both substrates. Interestingly, spermine and EDTA demonstrated similar effects on both PTPase enzymes; these compounds were moderately inhibitory to RCML dephosphorylation but stimulatory to dephosphorylation of MBP.

Kinetic parameters were also determined for LAR and LAR-PTP2 using both substrates (Table 2). There was a close range of K_m values for these artificial substrates varying between 3 and 8 μM. The turnover numbers calculated for MBP were 4 and 7 s^{-1} for LAR-PTP2 and LAR respectively assuming a single catalytic centre for each cytoplasmic domain. For RCML, these values were substantially higher (78 and 150 s^{-1} for LAR-PTP2 and LAR respectively), indicating a marked catalytic preference of these enzymes for RCML as a substrate compared with MBP.

DISCUSSION

The present study was performed initially to characterize PTPases that occur in liver and have a potential role in the regulation of the phosphorylation state of growth factor receptors in this tissue. Our strategy follows data from several laboratories which have demonstrated that as much as 75–85% of the total insulin receptor PTPase activity is found in the particulate fraction of liver and that the highest specific enzyme activity is present in a glycoprotein fraction of solubilized liver membranes, suggesting that the PTPase that acts to dephosphorylate the insulin receptor in liver might itself be a plasma-membrane glycoprotein (Goldstein et al., 1991). Other work has supported this hypothesis, including studies with permeabilized adipocytes (Mooney and Anderson, 1989) and insulin receptor dephosphorylation in liver endosomes (Faure et al., 1992).

As the transmembrane PTPase LAR, or a closely related homologue, is predominantly expressed in liver tissue in the rat (Goldstein et al., 1991), we used the human LAR cDNA as a probe to screen for homologous PTPases in a rat liver cDNA library. Using this strategy, we have identified the full-length sequence for the rat homologue of LAR as well as a closely related unique member of the LAR PTPase family, called LAR-PTP2, which is transcribed from a unique rat gene. The relative abundance of the isolated cDNA clones suggests that the mRNAs from which they are derived are quite rare. On the basis of the number of independent clones isolated from multiple screenings of the original cDNA library, rat LAR mRNA apparently represents seven copies per million, similar in magnitude to the abundance of rat insulin receptor mRNA which we have cloned from the same library (Goldstein and Dudley, 1990). The LAR-PTP2 cDNA is quite rare in liver, and is present on average at only one copy per million.

Comparison of the amino acid sequences among the PTPases isolated in this report provides further evidence for a family of transmembrane PTPases in human and rodent tissues that are closely related to LAR, including LAR-PTP2 and PTP-δ (Krueger et al., 1990; Mizuno et al., 1993). As described above, it is of interest that Southern-blot analysis using the conserved PTPase domain cDNA probe for human LAR and *Hin*dIII-

restricted rat genomic DNA identified only two genomic segments that appear to be derived from the genes for rat LAR and LAR-PTP2 respectively (Figure 4). Since a third fragment corresponding to a potential rat gene for PTP-δ was not observed, these findings suggest that perhaps LAR-PTP2 and PTP-δ arose as a result of divergent molecular evolution from an ancestral LAR-related PTPase homologue that arose initially by gene duplication.

The extracellular domains of rat LAR and LAR-PTP2 share several common structural features with the human PTPases, LAR, PTP-β and PTP-δ, and the *Drosophila* homologues, DPTP and DLAR (Streuli et al., 1989). These include regional homologies to fibronectin type III domains and the related N-CAM structure (Figure 2). These structural determinants suggest that these transmembrane 'receptor-like' PTPases may be regulated by the homotypic interactions that are characteristic of cell adhesion molecules. Unique regions in the extracellular domains of these enzymes may interact with other cell-surface molecules or specific circulating ligands which may exert a regulatory influence on the activity of the cytoplasmic PTPase domains, as has been demonstrated in studies with recombinant epidermal growth factor receptor–CD45 chimaera (Desai et al., 1993).

The expression of an alternatively spliced form of LAR-PTP2 (LAR-PTP2B) may also have tissue-specific functional effects, as this protein apparently lacks three of the eight fibronectin type III domains found in the extracellular domain of the full-length LAR-PTP2 expressed in liver. This sequence variation may have functional significance for the expression of LAR-PTP2 in various brain cell types or in its regional distribution. Alternative splicing of receptor-type PTPase mRNAs has been noted to affect both the extracellular and cytoplasmic domains (Streuli et al., 1987; Matthews et al., 1990; Barnea et al., 1993; Mizuno et al., 1993). During preparation of this paper, a report appeared documenting an additional splice variant of LAR-PTP2B that differentially uses polyadenylation sites leading to a truncated cytoplasmic segment lacking the second PTPase domain (Pan et al., 1993). Also, mapping of LAR-PTP2B in mRNA in brain by *in situ* hybridization has demonstrated high levels of expression in neurons localized to the olfactory neuroepithelium (Walton et al., 1993).

Streuli et al. (1992) and Yu et al. (1992) have shown that the extracellular domain of LAR is post-translationally processed by proteolytic cleavage and is non-covalently associated with the transmembrane segment in transfected cells. Further studies by these authors also demonstrated that a conserved penta-arginine sequence at the distal end of the extracellular domain of LAR is likely to function as a proteolytic processing site. This site is fully conserved in the rat and human LAR sequences, and a similar sequence (RKRR) appears in the same region of human PTP-δ. A similar polybasic motif is absent from the LAR-PTP2 protein, suggesting that LAR-PTP2 may have a different biosynthetic itinerary than LAR, and exist as an intact transmembrane PTPase with a large extracellular domain. Alternatively, LAR-PTP2 may be recognized by an enzyme with a unique specificity than can proteolytically process the extracellular domain in a fashion similar to LAR.

Expression of the recombinant cytoplasmic domains of rat LAR-PTP2 and LAR in a bacterial system revealed that they encode active PTPase enzymes. In *in vitro* studies, the catalytic domains of rat LAR and LAR-PTP2 demonstrated a lack of specificity for the two holoreceptor substrates, whose autophosphorylated tyrosine residues occur in completely different sequence contexts. These results are similar to the dephosphorylation of raytide and MBP by recombinant catalytic domains of a series of transmembrane PTPases including LAR, PTP-α, PTP-β and PTP-δ as reported by Krueger et al. (1990), where the ratio of activity between the peptide and protein substrates among these PTPases varied only between 0.28 and 0.33. However, there may be site-specific differences in the dephosphorylation of individual sites by LAR and LAR-PTP2 in the multiply phosphorylated protein substrates, as we have also demonstrated *in vitro* for the cytoplasmic domain of rat LAR compared with rat LRP (PTP-α) and PTPase1B (Hashimoto et al., 1992a).

Kinetic parameters calculated for homogeneously purified LAR and LAR-PTP2 showed they have high turnover numbers for both MBP and RCML (Table 2). LAR had a higher specific activity with linear reaction rates than LAR-PTP2, although the K_m values were also slightly higher for LAR. The calculated turnover numbers for LAR of 7 s^{-1} for MBP and 150 s^{-1} for RCML are similar to those reported for the rat LAR cytoplasmic domain of 3–6 s^{-1} using *p*-nitrophenyl phosphate or phosphorylated angiotensin I respectively (Pot et al., 1991) and 20–70 s^{-1} in experiments using a variety of non-radioactive phosphotyrosyl peptides (Cho et al., 1991, 1992).

As the intracellular domains of several of the transmembrane PTPases appear to have functional similarities, it is likely that their potential *in vivo* substrate specificity and their physiological roles are determined by the specificity of their tissue expression, as well as the structure of the extracellular domains in the intact proteins which may affect their interaction with cell surface proteins or regulatory ligands. Rat LAR and PTP-2 have strikingly divergent sequences in the region immediately surrounding the transmembrane domain and in the extracellular domain which may influence the function of these proteins in intact cells. Furthermore, subtle variations in the catalytic sequence of these PTPases, or the possible influence of specific cellular protein PTPase inhibitors (Ingrebritsen, 1989), may also direct their specificity or activity towards their actual physiological substrates *in vivo*. Further studies involving eukaryotic expression of various membrane-linked and soluble forms of the cloned PTPases in appropriate host cells will allow these hypotheses to be tested more directly.

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